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Reporting Summary

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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a	Confirmed	
☐	☒	The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
☐	☒	A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒	The statistical test(s) used AND whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
☐	☒	A description of all covariates tested
☐	☒	A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐	☒	A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☐	☒	For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☒	☐	For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐	For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☒	☐	Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	The light microscopy camera was run on SlideBook 6. The electron microscopy camera was run on AMT Capture v6
Data analysis	The electron microscopy images were randomized using a custom R script (R Studio 1.3, R version 3.5.1). Features were manually segmented in Fiji (version 1.0) using a custom plugin. Features were quantified in MATLAB versions R2017-R2020a using custom scripts. Statistical analysis and data visualization were performed in GraphPad Prism 6, 7, and 8. Custom R, MATLAB, and Fiji scripts are available upon request, and are the subject of a manuscript currently in preparation.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

Full data tables underlying the figures are available at https://figshare.com/authors/Shigeki_Watanabe/9106865 and in the Source Data. Raw images and image analysis files are available upon request.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	Power analysis was not used to determine the number (n) of synaptic profiles (2D EM) or serial-sectioning reconstructed active zones (3D EM). Our threshold of n >200 (from N = 2 or more experiments) for synaptic profiles was taken from previous work (Watanabe et al., 2013), based on 15-20% of synapses containing endocytic or exocytic events, such that >30 synapses with endocytic or exocytic events would be captured. For 3D EM, at least 30 active zones for each condition were reconstructed so that at least ~10 synapses with fusion events would be captured per replicate (based on prior data that ~30% of synapses respond to an action potential; Watanabe et al., 2013).
Data exclusions	Images that could not be reliably segmented, either because the image was not of a bona fide synapse or morphology was too poor, were excluded from segmentation; this was done only after randomizing the images. No other data were excluded.
Replication	All experiments were performed at least twice, and in most cases three times (from separate litters, different rounds of neuronal cell culture, frozen and processed separately, and segmented in separate batches of randomized images). While statistical analysis was performed on the data from all experiments pooled together, in each case similar results were obtained in each experiment. Furthermore, the DMSO controls shown in Figure 4 effectively serve as replicates of the no-stim, 5, and 11 ms time points from the experiments in Figure 3, and similar results were obtained for all metrics, except that 1) the number of pits at the 11 ms time point was greater in the DMSO controls and 2) fusions at 11 ms were concentrated at the center of the active zone in the DMSO controls as in the other experiments, but not as strongly, and 3) the number of docked vesicles was greater, though the proportional difference between the no-stim control and stimulated samples was similar.
Randomization	No randomization into experimental groups was performed prior to freezing, as different wells of the cell culture dish were presumed identical prior to handling during the experiment. For image segmentations, images were always randomized before manual segmentation, except for 2 (out of N = 3) of the experiments shown in Figure 3 and the first replicate of the experiments show in Figure 2, in which case they were analyzed blind by a person who was not familiar with the design of the experiment. These were the earliest experiments, and we had not yet adopted randomization scripts for our images.
Blinding	In the first two (of N = 3) experiments in Figure 3, all serial-section imaging (Figure 2), and two of the replicates of the experiments described in Figure 4 and 5, the microscopist was blind to the different conditions, while in all other cases they were not. To limit bias, synapses were found by bidirectional raster scanning along the section at 93,000x, which makes it difficult to "pick" certain synapses, as a synapse usually takes up most of this field of view, and anything that appeared to be a synapse was imaged without close examination. Because features identified were subtle, and any visible synapse was imaged, the possibility for bias during imaging is limited, even without blinding. For all other aspects of the study, blinding is not applicable. Blinding or group allocation was not possible while freezing samples, as all differences between treatment groups (EGTA treatment, stimulation/time point) were performed during the experiments.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☒	☐ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☐	☒ Animals and other organisms
☒	☐ Human research participants
☒	☐ Clinical data
☒	☐ Dual use research of concern

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals	No experiments were performed using live animals, only primary cell cultures. E18 or P0 wild-type C57BL/6J mice of both sexes were used for all cell culture. The sex of newborn or embryonic pups cannot be identified, but cells for neuronal culture were pooled from

	all the mice in a litter, and so contained cells from mice of both sexes in each experiment.
Wild animals	This study did not involve wild animals
Field-collected samples	This study did not involve samples collected from the field.
Ethics oversight	All animal care was performed according to the National Institutes of Health guidelines for animal research with approval from the Animal Care and Use Committee at the Johns Hopkins University School of Medicine.

Note that full information on the approval of the study protocol must also be provided in the manuscript.